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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **342nd Meeting** *of the Biochemical Society was held in the Department of Agricultural Chemistry, University College of North Wales, Bangor, on* 15 *July* 1955, *at* 11.00 *a.m., when the following papers were read:*

COMMUNICATIONS

Constitution of an Amylopectin α-Limit Dextrin. By W. J. Whelan and B. J. Bines. (*Department of Chemistry, University College of North Wales, Bangor*)

Whelan & Roberts (1952) reported that amylopectin and glycogen are each converted by human salivary α-amylase into a mixture of maltose, maltotriose and a series of limit dextrins, the molecules of which contain both α-1:4- and α-1:6-glucosidic linkages. The smallest of these dextrins is a pentasaccharide. This has now been isolated from a hydrolysate of amylopectin (waxy maize starch) and subjected to structural analysis. As previously indicated (Whelan & Roberts, 1952) it consists of an α-maltose unit attached 1:6-glycosidically to one of the glucose members of a maltotriose fragment, but we have now established that the actual structure differs from that tentatively postulated inasmuch as the α-maltose unit is joined to the non-reducing end glucose member of the maltotriose unit and not to the central glucose member. The structure of the pentasaccharide is therefore defined as *O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→6)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-D-glucopyranose.

This conclusion is based on the following facts: (i) The de-branching enzyme, R-enzyme, hydrolyses the α-1:6-linkage in the dextrin, yielding maltose and maltotriose. (ii) The methylated dextrin yields 2:3:4-tri-*O*-methyl glucose among the products of hydrolysis. (iii) The results of periodate oxidation experiments show that neither the primary hydroxyl group in the reducing end unit of the pentasaccharide, nor that in the glucose unit penultimate to the reducing end, is involved in uniting the di- and tri-saccharide fragments.

A pentasaccharide from the same source has been examined by French, Nordin & Wild (personal communication from Dr Dexter French) using different methods. The conclusions of these authors agree with ours.

We thank Professor Stanley Peat, F.R.S. for his advice and encouragement.

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The α-, ω-, and (ω − 1)-Oxidation of *n*-Propyl- and *n*-Butyl-Benzenes in Rabbits. Determination of Hippuric Acid. By A. M. el Masry, J. N. Smith and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

Biological oxidation is known to occur in the ω-position of *n*-alkylbenzenes, and oxidation might be expected at the activated α-methylenic carbon atom. In rabbits, it is now shown that *n*-propyl- and *n*-butyl-benzenes are oxidized not only at the α- and ω-positions, but also at the penultimate or (ω − 1)-position. The latter type of oxidation occurs during the metabolism of certain barbiturates (Maynert, 1952) and in the *n*-butyl side chain of the antirheumatic drug, butazolidine (Burns *et al.* 1955).

In ethylbenzene, α- and ω-oxidation occurs (Smith, Smithies & Williams, 1954). Further proof of ω-oxidation has been obtained by the chromatographic detection of phenaceturic acid. Hippuric and phenaceturic acids are difficult to separate on paper. Trial of many solvents eventually gave separation in benzene/acetic acid/water (1:1:2) with R_F 0·18 for hippuric and R_F 0·28 for phenaceturic acid.

n-Propylbenzene yielded in rabbits hippuric acid (15% of dose), i.e. by ω- followed by β-oxidation, but no phenaceturic acid. (−)-Ethylphenylcarbinyl glucuronide (triacetyl methyl ester, m.p. and mixed m.p. 124°; $[\alpha]_D - 82°$ in $CHCl_3$) formed by α-oxidation, and small amounts of benzylmethylcarbinol (identified as the ketone 2:4-dinitrophenylhydrazone, m.p. and mixed m.p. 153°) formed by (ω − 1)-oxidation, were also isolated.

n-Butylbenzene yielded phenaceturic acid (10–15% by isolation) by ω- and β-oxidation, methylphenethylcarbinol (ketone 2:4-dinitrophenylhydrazone, m.p. and mixed m.p. 128°) by (ω − 1)-oxidation, and propylphenylcarbinol (ketone 2:4-dinitrophenylhydrazone, m.p. and mixed m.p. 194°) by α-oxidation.

Hippuric acid in urine (1 ml. containing 250 μg.) in the presence of phenaceturic acid was determined by extraction of the acidified urine (pH 2) with ethyl acetate (5 ml.). The extract (1 ml.) was evaporated to dryness at 110° and the residue dissolved in acetic anhydride (2 ml.) and heated at 120° with *p*-dimethylaminobenzaldehyde in acetic anhydride (1 ml. of 20%, w/v) (cf. Gaffney *et al.* 1954). Ethyl acetate (2 ml.) was added to the product and the colour measured at 460 mμ. Phenaceturic acid under these conditions gives a yellow colour with a very low absorption at 460 mμ. The excretion of hippuric acid after feeding a number of alkylbenzenes was determined.

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The Metabolism of Salicylohydrazide and Salicylohydroxamic Acid in Rabbits. By W. M. McIsaac and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W. 2*)

Salicylohydrazide and salicylohydroxamic acid and its 3-bromo-derivative are tuberculostatic (cf. Ma & Tien, 1953; Urbanski, Hornung, Slopek & Venulet, 1952); and we were interested in the biological stability of the hydrazide and hydroxamic acid groups.

Other work in this laboratory (el Masry, Smith & Williams, unpublished) had shown that benzohydrazide and benzohydroxamic acid were largely hydrolysed *in vivo* and excreted as hippuric acid. Benzohydrazide was also toxic in doses greater than 100 mg./kg. Salicylohydrazide and salicylohydroxamic acid, however, were well tolerated at 500 mg./kg. and were not readily hydrolysed to salicylic acid.

The main metabolite of salicylohydrazide was the corresponding phenolic *glucuronide* (m.p. 204–5°; $[\alpha]_D^{20} - 65°$); small amounts of salicylic and gentisic acids, and unchanged salicylohydrazide were also excreted, but there was no evidence of acetylation. The glucuronic acid conjugation was 55% of the dose, but there was no sulphate conjugation. The glucuronic acid conjugation of *N*-acetylsalicylohydrazide was higher, 70%, and the *N*-acetyl *glucuronide* was readily isolated from the urine (m.p. 208–210°; $[\alpha]_D^{20} - 63°$).

Salicylohydroxamic acid also formed a glucuronide (glucuronic acid conjugation, 49%, ethereal sulphate, 16%). The glucuronide was isolated as a non-crystalline gum which formed a glassy triacetyl methyl ester, m.p. 70–76° and $[\alpha]_D^{20} - 27°$ (in $CHCl_3$). 3-Bromosalicylohydroxamic acid, which has had a clinical trial in tuberculosis, is highly conjugated *in vivo* (glucuronic acid, 60%, ethereal sulphate 20%). The *glucuronide* was isolated (m.p. 217–218°; $[\alpha]_D^{20} - 75°$ in 0·1 N-NaOH).

The suggestions (*a*) that the differing toxicities of the benzo- and salicylo-derivatives studied are due to the differing stabilities *in vivo* of the hydrazide and hydroxamic acid groups, and (*b*) that the therapeutically active hydrazide and hydroxamic acid groups may be protected by providing the molecule with a group capable of undergoing an alternative metabolic reaction (i.e. conjugation) competing with hydrolysis, will be discussed.

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Observations on the Proteolytic Activity of Gastric Juice and Mucosal Extracts. By W. H. Taylor and J. R. P. O'Brien. (*Department of Clinical Biochemistry, Radcliffe Infirmary, University of Oxford*)

A potent 'cathepsin' in human gastric juice and swine mucosal extracts which hydrolyses edestin optimally at pH 3·3 has been described (Buchs, 1947, 1949, 1953), but its existence is denied by Geilenkirchen & Elbers (1951), who claim that the activity at pH 3·3 is an artifact attributable to the use of edestin as substrate; by Crebolder, Engel & Otten (1951), who find that an apparent optimum at pH 3·3 may result under experimental conditions from inactivation of pepsin at lower pH's; and by Tolckmitt (1954), who finds the optimum at pH 3·3 to be an artifact caused by changing from HCl to acetic acid for control of pH.

In experiments in which a formol titration method has been used for measuring proteolysis, human gastric juice and extracts of human, hog or

calf stomach show two well-marked pH optima at pH 1·6–2·4 and 3·3–4·0 when hydrolysing casein, plasma, serum albumin, bovine plasma albumin fraction V, edestin and gelatin. The two optima occur independently of changes of buffer solution. Only one optimum, at pH 1·6–1·8, was however found for egg albumin. Pepsin (Parke-Davis) showed only one pH optimum when digesting plasma and casein, but crystalline swine pepsin (Armour) showed two optima.

Chromatographic analysis on paper of the digestion mixtures obtained at pH 3·5 and 2·0 indicates differences in the course of digestion at the two optimal pH's. Attempts to separate two active proteolytic components have been made by fractionation with ammonium sulphate and sodium chloride, by adsorption on calcium phosphate gel and ion-exchange resins, by electrophoresis, and by precipitation from concentrated gastric juice at acid pH's. Whenever solutions showing one peak of activity were obtained, no evidence of a second peak remained in the gastric juice or mucosal extracts. More usually the two peaks remained together. Separation of two active components was thus never achieved.

It is concluded that gastric juice and mucosal extracts digest many proteins as readily at pH 3·2–4·0 as at 1·6–2·4, that the existence of a separate cathepsin is doubtful, and that the optimal activity at pH 3·2–4·0 may be a property of pepsin.

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Enzymic Reactions of Erythrose 4-Phosphate. By H. L. Kornberg* and E. Racker. (*Public Health Research Institute of the City of New York Inc., New York, U.S.A.*)

The occurrence of erythrose 4-phosphate as an intermediate in carbohydrate metabolism was indicated by recent studies of the mechanism of action of transaldolase (Horecker & Smyrniotis, 1953, 1955; Horecker, Smyrniotis, Hiatt & Marks, 1955) and transketolase (Racker, de la Haba & Leder, 1954). However, this substance had not been isolated, and a direct demonstration of its participation in the metabolism of carbohydrate had not been made.

In the present work, erythrose phosphate was formed by the reaction

fructose 6-p + glyceraldehyde → erythrose-p + ribulose, (i)

and precipitated as a barium salt (Srere, Kornberg & Racker, 1955). This preparation was compared with synthetic D-erythrose 4-phosphate (Ballou, Fischer & MacDonald, 1955), a sample of which latter compound was kindly donated by these investigators. Both materials behaved identically on paper chromatography, and the following reactions have been shown to occur with both the enzymically produced and the synthetic compounds:

fructose 6-p + erythrose 4-p → heptulose 7-p + triose-p, (ii)

ribulose 5-p + erythrose 4-p → fructose 6-p + triose-p, (iii)

hydroxypyruvate + erythrose 4-p → fructose 6-p($+CO_2$), (iv)

heptulose 7-p + erythrose 4-p → fructose 6-p + ribose 5-p, (v)

erythrose 4-p + DPN^+ → phospho-erythronic acid (?) + DPNH + H^+. (vi)

Reactions (i), (iii), (iv) and (v) were catalysed by crystalline transketolase (de la Haba, Leder & Racker, 1955), (ii) by highly purified transaldolase (Srere, 1954; Srere *et al.* 1955) and (vi) by crystalline glyceraldehyde 3-phosphate dehydrogenase (Cori, Slein & Cori, 1948). The identity of the enzymically produced erythrose phosphate was further established by a colorimetric test of Dische (1955) and by the formation of a compound, behaving identically with authentic D-erythrose on paper chromatography, on treatment with prostatic acid phosphatase.

These findings thus indicate that the enzymically produced erythrose phosphate was identical with the synthetic D-erythrose 4-phosphate. They further suggest that this compound may play an important role in carbohydrate metabolism, as was indicated by recent work on the path of carbon in photosynthesis (Bassham, Benson, Kay, Harris, Wilson & Calvin, 1954), on the mechanism of the glucose 6-phosphate shunt (Horecker, 1953; Racker, 1954) and on the biosynthesis of shikimic acid (Davis, 1955).

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Estimation of Lower Aliphatic Amines. By L. Ekladius and H. K. King. (*Department of Biochemistry, University of Liverpool*)

During investigations on bacterial amino acid decarboxylases (King, 1953) a method was required for estimating aliphatic amines in the presence of ammonia. Chloro-2:4-dinitrobenzene (DNCB) reacts with the lower primary aliphatic amines to yield *N*-alkyldinitroanilines. These derivatives, unlike the parent compounds, have an intense yellow colour ($\lambda_{max.}$ 358 mμ.; ϵ 17 400 for *n*-butyl dinitroaniline). Under suitable conditions DNCB will not react with ammonia. This reaction proved suitable for assay of the amines by the following procedure. The solution for assay is made alkaline (NaOH) and steam-distilled in the micro-Kjeldahl apparatus of Markham (1942). The distillate is collected in a few ml. of *n*-propanol, made up to a suitable volume (10 ml.) with propanol, and a solution (5 ml.) of DNCB (0·1% in propanol; stored in dark) is added. A considerable excess of DNCB is required as the reaction apparently proceeds to an equilibrium not favourable to the product. The mixture is allowed to stand in a covered tube for 15 min. in a cold water bath, which is then heated to 85° for 30 min. and to 100° for a further 30 min. This procedure is necessary to avoid loss of the volatile amines. A blank and a series of standards are prepared at the same time; assay and standards are read by comparison with the blank in a spectrophotometer at 358 mμ. (or at 450 mμ.; the DNCB has no absorption at this wavelength). The EEL colorimeter with a 621 (blue) filter may also be used. The useful range of the method is 50–200 μg. amino-N. All the amines examined, including *n*-butyl-, *iso*amyl-, and phenylethyl-amines, give identical colour intensities. Ammonia, even in large excess, yields no colour.

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The Free Amino acids of Fish. *Post-mortem* Changes in Sterile Skeletal Muscle of North Sea Codling (*Gadus callarias*). By W. Hodgkiss and N. R. Jones. (*Torry Research Station, Food Investigation Organization, Department of Scientific and Industrial Research*)

Earlier studies (Jones, 1954*a*, 1955*a*) showed that the free amino acid composition of fresh codling muscle varies seasonally and that storage of codling in ice leads to changes resulting from the combined actions of leaching, invasion by bacteria and autolysis.

Bruns (1909) found that flesh of healthy, freshly caught fish is bacteriologically sterile. In an attempt to ascertain the contribution of autolysis to these changes, blocks of muscle have been dissected from freshly killed codling under aseptic conditions and incubated in sterile dishes at 0°. Muscle, shown to be bacteria-free by subculture into fluid and on to solid media, was homogenized into 75% ethanol for chromatographic evaluation as described in a previous communication (Jones, 1954*b*).

Serial chromatograms showed that, compared with iced fish, little change occurred in most free amino acids. Individuals varied somewhat in the extent of *post-mortem* change but the overall patterns were similar in batches examined at and after the spawning period. Initial compositions varied from batch to batch in the normal seasonal

manner. For a typical individual in March, glutamic acid rose from 10 to 29 mg./100 g. muscle after 25 days' incubation. β-Alanine and 1-methylhistidine levels rose similarly from 2·5 and 6·2 mg. to 66 and 101 mg./100 g. respectively. Free alanine fell from 16 to 5 mg./100 g. Lysine and leucine similarly decreased from 6·5 and 5·2 mg./100 g. to <0·4 mg. These changes were continuous through the 25-day incubation period, but it is likely that the actions of bacterial proteolysis and leaching of salts and protein encountered during normal spoilage in ice would substantially alter muscle enzyme systems. Taurine, glycine and other amino acids remained constant in level.

Of these values only those of β-alanine and 1-methyl-histidine compare with findings for codling stored in ice. The present study indicates that, with the notable exception of anserinase action (Jones, 1955*b*) and a certain glutamic acid liberation, most changes in spoiling iced codling derive from bacterial and leaching actions. The *post-mortem* changes resulting in uptake of some free amino acids by muscle systems are nevertheless of considerable interest.

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The Fluorimetric Determination of β-Glucosidase. By D. Robinson and R. T. Williams. (*Department of Biochemistry, St Mary's Hospital Medical School, London, W.* 2)

In a previous communication from this laboratory (Mead, Smith & Williams, 1954) a highly sensitive fluorescence method for the assay of β-glucuronidase with 4-methylumbelliferone β-D-glucuronide was described. This method has now been adapted to the assay of β-glucosidase. 4-*Methylumbelliferone* β-D-*glucoside* (m.p. 211° and $[\alpha]_D$ −89·5° in water) was synthesized and found to be almost non-fluorescent after purification.

For the assay of the enzyme, 0·0005 M solutions of the β-glucoside were used as substrate in 0·14 M acetate buffer at pH 5·2 with an incubation time of 0·5 hr. which must be accurately measured. The quantities of enzyme, substrate and buffers used were similar to those described for the assay of β-glucuronidase (Mead *et al.*, 1954) and the liberated 4-methylumbelliferone was determined fluorimetrically in a glycine buffer at pH 10·3.

Locust crop fluid was found to contain high amounts of β-glucosidase varying from 5800 to 960 000 (average for 8 samples, 272 000) expressed as μg. 4-methylumbelliferone liberated/ml./hr. at pH 5·2. A sample of commercial emulsin gave 36 000 μg./g./hr. at pH 5·2. For cockroach (*Periplaneta americana*) gut tissue the following average values were obtained at pH 5·4: fore-, 120 000, mid-, 260 000, and hind-gut, 38 000 μg./g./hr.

A sample of commercial 'bacterial β-glucuronidase' was found to contain β-glucosidase, 180 000 μg./g./hr. with an optimum pH of 4·6 and β-glucuronidase, 46 000 μg./g./hr. with an optimum pH of 4·2. A sample of locust crop liquor containing β-glucosidase at 960 000 μg./ml./hr., also contained β-glucuronidase at 160 000 μg./ml./hr. The β-glucuronidase could be distinguished from the β-glucosidase by inhibiting the former enzyme with boiled saccharate, and when both substrates (glucuronide and glucoside) were added to the crop liquor, the effect of each enzyme was additive.

Potassium 4-methylumbelliferone sulphate was found to be unsuitable for the fluorimetric determination of arylsulphatase since it is appreciably fluorescent, although it was about 230 times less fluorescent than 4-methylumbelliferone. It was hydrolysed optimally by the arylsulphatase of takadiastase at pH 5·4–5·8.

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The Metabolism of the Trichloronitrobenzenes in the Rabbit. By H. G. Bray, Sybil P. James and W. V. Thorpe. (*Physiology Department, Medical School, University of Birmingham*)

In a previous communication (Bray, James & Thorpe, 1955) it was reported that mercapturic acids were formed in the rabbit after administration of 2:4-, 2:5- and 3:4-dichloronitrobenzenes. In this process a chlorine atom was eliminated and it was shown that the reaction was, in effect, an acetylcysteyldechlorination involving a relatively labile (Berckmans & Holleman, 1925) chlorine atom. Further examples of such a reaction have been encountered in a study of the metabolism of the six trichloronitrobenzenes in the rabbit. At least four of these yield dichloronitromercapturic acids

and the mercapturic acids have been isolated from the urine of rabbits dosed with 2:3:4-, 2:3:5-, 2:4:5- and 3:4:5-trichloronitrobenzene. All the trichloronitrobenzenes undergo reduction and a significant percentage of the dose is excreted as the corresponding trichloroaniline. Phenolic metabolites are excreted conjugated with glucuronic and sulphuric acids. The amounts of mercapturic acid, trichloroaniline, glucosiduronic acid and ethereal sulphate excreted in urine accounted for more than 75% of the dose of all the isomers except 2:4:6-trichloronitrobenzene (65%). An azoxybenzene has been isolated from the urines of animals dosed with 2:4:5-trichloronitrobenzene.

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A Vitamin A Isomer in the Eyes of *Meganyctiphanes norvegica*. By L. R. Fisher, K. M. Henry, S. K. Kon, P. A. Plack and S. Y. Thompson. (*National Institute for Research in Dairying, University of Reading*)

We have already reported a discrepancy between the chemical and biological assay of vitamin A in the euphausiid crustacean *Meganyctiphanes norvegica* (Fisher, Kon & Thompson, 1952; Kon, 1954), the biological value being about 60% of that by the Carr–Price test. The cause of this discrepancy has since been investigated and some findings reported (Anonymous, 1955).

Twenty kilograms of *Meganyctiphanes* were extracted, saponified and chromatographed by our usual methods (Fisher *et al.* 1952). Fractional chromatography on alumina produced a concentration in the first fractions of a component with an absorption peak at 315 mμ. in *n*-hexane, and in the last fractions of another component absorbing maximally at 325 mμ. Biological assay of '315' with rats gave a potency of 60% of that of the starting material, which itself would be 60% as active as all-*trans* vitamin A.

In a further separation of these two components by fractional chromatography, the starting material was 76 g. of eyes of *M. norvegica* derived from some 20 000 specimens. The '325' component in the last fractions, from its absorption curve, and that of the retinene from it, was all-*trans* vitamin A. The first few fractions showed a peak at 315 mμ. as before, but with further purification by repeated fractional chromatography after precipitation of sterols, a product absorbing maximally at 311–312 mμ. in *n*-hexane was obtained.

This substance gave a blue colour in the Carr–Price reaction, with an absorption maximum at 618 mμ., and formed anhydro-vitamin A with 0·06N ethanolic HCl, but at a slower rate than all-*trans* vitamin A. An *n*-hexane solution, shaken with a crystal of iodine in daylight for some 30 sec. showed a change of the peak from 311 to 325 mμ., and an increase of extinction at the maxima (measured at 311 and 325 mμ. respectively) of 36%. This product seemed to be all-*trans* vitamin A.

These properties of '315' are consistent with its being a *cis* isomer of vitamin A. Wald & Burg (1955) recently found neovitamin Ab in lobster eyes, and further characterization of the *Meganyctiphanes* compound is in progress in collaboration with Professor Wald.

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The Prevention of Swelling of Mitochondria. By A. Fonnesu and R. E. Davies. (*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry, University of Oxford*)

Previous work has shown that isolated mitochondria can maintain a low water content in conditions favourable to oxidative phosphorylation (cf. Harman & Feigelson, 1952*a*, *b*; Macfarlane & Spencer, 1953; Raaflaub, 1953; Brenner-Holzach & Raaflaub, 1954; Bartley & Davies, 1952, 1954; Price & Davies, 1954). Conditions leading to prevention of swelling have now been analysed.

Rat-liver mitochondria were prepared at 0° in 0·25M sucrose as described by Macfarlane & Spencer (1953) and washed twice with 0·25M sucrose. After incubation in various media for

30 min. at 28° in a Dubnoff shaker, the mitochondria (5 mg. dry wt./ml.) were separated at 25 000 *g* for 4 min. at 20°.

In a medium containing 20 mM glutamate, 2 mM-$MgSO_4$, 10 mM phosphate buffer, pH 7·4 and 80 mM sucrose mitochondria swelled (13% dry wt.) much more in the absence than in the presence (20% dry wt.) of 1 mM adenosine-5′-phosphate (AMP) (cf. Macfarlane & Spencer, 1953).

Glutamate or other added substrate was found not to be necessary for the prevention of swelling. In this condition, anaerobiosis, 4 mM-KCN or 0·01, 0·1 or 1 mM 2:4-dinitrophenol actually reduced the swelling occurring in the absence of AMP, but partially inhibited the effect of AMP.

Mitochondria incubated in 40 mM sucrose containing 2 mM-$MgSO_4$ were less swollen (15% dry wt.) than when 10 mM phosphate was also present (11% dry wt.), whilst both 40 mM sucrose and 40 mM sucrose containing 10 mM phosphate gave a mitochondrial dry wt. of 10%. AMP (1, 3 or 5 mM) largely prevented swelling in sucrose alone (18% dry wt.), sucrose plus Mg^{2+} (19% dry wt.), sucrose plus Mg^{2+} plus phosphate (17% dry wt.) but not in sucrose plus phosphate (10% dry wt.).

Since AMP can prevent swelling even in very dilute sucrose under conditions unfavourable to oxidative phosphorylation, other compounds (all 1 mM) were tested for this activity. Adenosine-diphosphate (ADP) was less effective than AMP but better than AMP plus 1 mM phosphate. Adenosine-triphosphate was less effective than ADP but better than AMP plus 2 mM phosphate. The following were ineffective; adenosine-3′-phosphate, adenosine, inosine-5′-phosphate, inosine, uridine-5′-phosphate and flavin mononucleotide; of other compounds ethylenediaminetetra-acetate was better (21% dry wt.) than AMP; cysteine, glutathione, citrate and oxalate were less effective than AMP but sulphate, chloride and 2:3-dimercaptopropanol had no significant effect. Mn^{2+} was better (19% dry wt.) than Mg^{2+}; Ca^{2+}, Na^{+}, K^{+} had negligible effect. Whilst Mn^{2+} and Mg^{2+} potentiated the action of AMP, Ca^{2+} antagonized both AMP and AMP plus Mg^{2+}.

It therefore seems unlikely that oxidative phosphorylation is necessary to prevent swelling. Simple osmotic effects appear to be excluded and it is possible that the agents preventing swelling act directly on the structure of the mitochondria.

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The Chemical Structure of Pepsin-modified Cytochrome *c*. By G. Leaf and N. E. Gillies.
(*Department of Biochemistry, University of Glasgow*)

Tuppy & Paleus (1955) have recently published the following structure for the peptic degradation product from cytochrome *c* (PMc) first described by Tsou (1951):

Val.Glu(NH_2).Lys.CyS.Ala.Glu(NH_2).CyS.
His.Thr.Val.Glu.

The prosthetic group is attached to the sulphur atoms of the two cysteine residues.

Work simultaneously carried out in this laboratory largely confirmed their findings. Our preparation had a similar amino acid composition to that of Tuppy & Paleus, but contained rather more lysine and a small amount of glycine. Valine was the only detectable *N*-terminal group indicated by the methods of Sanger (1945) and Edman (1950), and examination of partial hydrolysates of PMc yielded results supporting the same amino acid sequence between the *N*-terminal valine residue and the threonine residue.

After removal of the prosthetic group from PMc by incubation with mercuric acetate at least two peptide fractions can be separated. One of these contains histidine and threonine and little or no lysine, the other contains lysine but no histidine or threonine. This is found whether the peptide is purified by paper electrophoresis or by partition chromatography as described by Tuppy & Paleus (1955). At first sight this suggests that the structure may be more correctly represented as two peptide chains linked together by the prosthetic group. Alternative hypotheses are that the purified PMc is still heterogeneous, containing two overlapping

fragments of the same peptide sequence, or that incubation with a heavy metal to remove the prosthetic group causes fission of bonds in addition to the thioether linkages between peptide and prosthetic group.

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The Bracken Poisoning of Ruminants. By A. J. Thomas, J. E. Watkin, I. A. Evans and W. C. Evans. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

Field outbreaks of bracken poisoning in livestock occur in horses and cattle, but the clinical symptoms differ appreciably in the two species.

Bracken 'staggers' in the horse is a thiamine deficiency (Evans, Evans & Roberts, 1951) caused by a thiaminase in bracken fern (Jones & Evans, 1952). The condition responds to thiamine therapy.

Cattle bracken poisoning is characterized by haemorrhage and pyrexia with a severe leucopenia and thrombocytopenia due to depression of bone marrow activity. The tissues are not unduly deficient in thiamine. No therapeutic agent so far tried allays the course of the disease, which is almost invariably fatal (Evans, Evans & Hughes, 1954).

Steaming the bracken plant abolishes its toxicity for all species. This treatment inactivates thiaminase, but it has been uncertain whether this enzyme is concerned in the depression of bone marrow activity, the essential lesion in cattle bracken poisoning.

Fresh bracken fronds were minced and extracted with water in the cold at pH 8. The clarified whole extract was separated into protein and non-protein fractions by cold isoelectric precipitation at pH 3. High thiaminase activity was retained in the reconstituted protein fraction whilst the non-protein supernatant fraction was completely dialysable.

These fractions, each equivalent to 10 lb. of fresh fronds, were introduced daily by rumen fistula into sheep receiving a free supply of hay. The sheep were bled weekly for haematological investigations. During the whole experiment the sheep receiving the protein fraction (containing thiaminase) showed normal values for all blood elements, although in total it had received the enzyme from about half a ton of fresh bracken.

The sheep receiving the non-protein fraction showed a rise and fall in leucocytes and platelets and a variation in differential count similar to the observed blood changes in cattle (Evans *et al.* 1954) during the earlier stages of poisoning by whole bracken.

Although the supply of fresh bracken became exhausted before the acute stage of poisoning was reached, the results suggest that the factor toxic to the bone marrow of ruminants is a soluble, small molecular substance distinct from thiaminase.

Sheep are more resistant to the toxic property of bracken than are cattle. A similar experiment using cattle is now in progress, which should give a more clear cut result.

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An Extracellular Polysaccharide from *Mucor racemosus*. By L. Hough and M. B. Perry. (*Department of Organic Chemistry, University of Bristol*)

Mucor racemosus (Commonwealth Mycological Culture Collection No. 17,364), when grown on a medium containing 5% D-glucose and Czapek-Dox mineral salts, produces a mixture of soluble polysaccharides (containing a glucan) from which the major component was separated by fractional precipitation with ethanol. This polysaccharide ($[\alpha]_D^{20} - 51°$ (*c*, 1·0 in water); N, 0·56% = approx. 3·5% protein; sulphated ash, 1·3%) was examined in the Tiselius electrophoresis apparatus using borate buffer, pH 9·2, and phosphate buffer, pH 7·0 (Northcote, 1954); in each case the single ascending and descending peaks were enantiographic, suggesting that the polysaccharide was homogeneous. Aqueous solutions of the polysaccharide were acidic (equiv. wt., 349), gave no colour with iodine, and were non-reducing to Fehling's solution. Hydrolysis with 98% formic acid at 100° for 20 hr., followed by paper chromatography (Hough, Jones & Wadman, 1950) indicated the presence of glucuronic acid, galactose, mannose, and fucose. Column chromatography

(Hough, Jones & Wadman, 1949) afforded crystalline specimens of D-galactose, D-mannose, L-fucose, and D-glucurono-6→3-lactone. The polysaccharide contained 58% hexuronic anhydride (method of Johansson, Lindberg & Theander, 1954), and hydrolysis with formic acid gave 4·5% D-galactose, 6·8% D-mannose, and 13·4% L-fucose (calculated as anhydrosugars) (Jones & Pridham, 1954). Thus the polysaccharide is composed approximately of D-galactose (2), D-mannose (3), L-fucose (6), and D-glucurono-6→3-lactone (24 parts).

Autohydrolysis of a 1% aqueous solution (pH 2·6) of the polysaccharide at 80° for 9 hr. liberated all of the 6-deoxyhexose, suggesting that in the polysaccharide, L-fucose is present in the furanose form as non-reducing end groups.

Periodate oxidation of the sodium salt of the polysaccharide under unbuffered and buffered conditions (pH 3·6) (Neumüller & Vasseur, 1953) was studied. During the oxidation L-fucose was completely destroyed, supporting the suggestion that it occupies non-reducing terminal positions. Further study of periodate oxidation of the polysaccharide and of hydrolysis products of the methylated polysaccharide is in progress.

It is believed that this is the first characterization of D-glucuronic acid and L-fucose as products from non-pathogenic fungi.

We thank the Director and Staff of the M.R.C. Antibiotics Research Station, Clevedon, for their help in growing the organism, and to the University of Bristol for the award of a Post-graduate Scholarship to one of us (M.B.P.).

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The Production of Indoleacetic Acid by Bacteria. By B. B. Stowe. (*Department of Agricultural Chemistry, University College of North Wales, Bangor*)

The study of the conversion of tryptophan to the plant growth hormone, indoleacetic acid (IAA), is difficult in higher plants since only minute amounts of the hormone are normally synthesized. Certain bacteria have long been known to produce IAA in large quantities (Salkowski, 1885); a study of such bacteria has yielded information on the pathway of synthesis of IAA, and on analytical methods, which should prove useful in studies of the higher plant enzyme systems.

Pure cultures of bacteria grown in liquid media containing glucose or sucrose, salts, and 0·1% tryptophan were extracted with ether and the extract examined by paper chromatography. Three micro-organisms, *Escherichia coli* K_4, *Pseudomonas fluorescens* A 3/9, and *Agrobacterium tumefaciens* NCIB 8150, were found to produce large quantities of IAA, identified by its R_F and colour reactions (Stowe & Thimann, 1954).

From large-scale cultures of these organisms stable freeze-dried preparations are obtainable. Homogenization yields cell-free extracts, and in two cases further purification is accomplished through the use of calcium phosphate gel and ammonium sulphate precipitation. In all three organisms, the major activity of the cell-free extract is a vigorous transamination of tryptophan which is completely dependent on any one of several α-keto acids, and which in all cases also shows strong stimulation by pyridoxal phosphate. The production of indolepyruvic acid (IPyA), IAA, and the appropriate amino acid can be demonstrated on chromatograms.

Pure synthetic IPyA, when chromatographed similarly, slowly decomposes spontaneously to IAA and other unidentified products. Furthermore, as in the case of *p*-hydroxyphenylpyruvic acid (Painter & Zilva, 1947), the pure compound crystallizes in the enol form and in solution tautomerizes within 20 min. at pH 8 and 25° largely to the keto form. The enol may be detected by 2:6-dichlorophenolindophenol reduction and the ketone by the total hydrazones determination (Friedemann & Haugen, 1943). Other unknown changes in solution occur, the final equilibrium not being reached until 4 hr. after dissolving the compound, as is indicated by both the total hydrazones and Salkowski (Gordon & Weber, 1951) reagents. Also, IPyA interferes with the quantitative Ehrlich test for indoles (Spies & Chambers, 1948). These complications have prevented an accurate assessment of the extent of enzymic involvement in the oxidative decarboxylation of IPyA.

I thank Professor W. C. Evans for his interest and the use of laboratory facilities; Drs H. Saz and W. J. Whelan for bacterial cultures; and Drs A. Ek, K. Farrar, and G. Smith for samples of IPyA. This work was carried out during the tenure of an U.S. National Science Foundation Fellowship.

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The Structure of Laminarin: Isolation of a Non-reducing Trisaccharide. By STANLEY PEAT, W. J. WHELAN, H. G. LAWLEY and J. M. EVANS. (*University College of North Wales, Bangor*)

Peat, Whelan & Lawley (1955) reported the presence of mannitol in the 'insoluble' and 'soluble' forms of the seaweed polysaccharide laminarin, previously thought to contain only glucose. Fractionation of a partial acid hydrolysate of insoluble laminarin (137 g.) gave mannitol and 1-*O*-*β*-D-glucopyranosyl-D-mannitol (crystalline *benzoate*, m.p. 88–94°; $[\alpha]_D + 39{\cdot}1°$ in $CHCl_3$) as components of the mono- and di-saccharide fractions. The latter compound has also been obtained from soluble laminarin.

We now report the isolation of a non-reducing trisaccharide (approx. 1·1 g.) from the same hydrolysate. Qualitative examination of the products of partial acid and enzymic (*β*-glucosidase) hydrolysis indicated this substance to be 3-*O*-*β*-D-*glucopyranosyl*-1-*O*-*β*-D-*glucopyranosyl*-D-*mannitol* (1-*O*-*β*-laminaribiosylmannitol). Confirmation of this structure is provided by the following evidence:

(1) *Mannitol content.* 0·98 mol. prop. mannitol (measured by an unpublished method).

(2) *Periodate oxidation.* Oxidized at 15° with sodium metaperiodate, the trisaccharide consumed 6·08 mol. prop. periodate and yielded 3·72 mol. prop. formic acid and 1·03 mol. prop. formaldehyde. Calculated values are 6·0, 4·0 and 1·0 mol. prop., respectively.

(3) *Synthesis.* The trisaccharide has been synthesized by the following sequence of reactions:

hepta-*O*-acetyl-α-laminaribiosyl bromide + 1:2:3:4-tetra-*O*-acetyl-*β*-D-mannose → undeca-*O*-acetyl-6-*O*-*β*-laminaribiosyl-*β*-D-mannose

(1) De-acetylation
(2) $NaBH_4$ → 1-*O*-*β*-laminaribiosylmannitol.

The synthetic and laminarin-derived compounds respectively show $[\alpha]_D - 24{\cdot}3°$ and $-24{\cdot}8°$ in water. Each gives a crystalline *dodeca-acetate*, m.p. and mixed m.p., 144–145° (uncorr.); $[\alpha]_D^{20} - 17{\cdot}9°$ and $-18{\cdot}6°$ in $CHCl_3$, respectively. (Found: acetyl, 51·4. Calc. for $C_{42}H_{58}O_{28}$, 51·1%.)

Only trace amounts of other mannitol-containing trisaccharides have been detected in the laminarin hydrolysate. In particular, less than 40 mg. of 1:6-di-*O*-*β*-glucosylmannitol was obtained. (This compound, together with 1-*O*-*β*-glucosylmannitol, was found by Lindberg (1953) in an aqueous extract of *Fucus vesiculosus*.) We therefore conclude that in those laminarin molecules which contain mannitol the hexitol residues are mainly, if not exclusively, located at the chain ends.

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The Early Intermediate formed in the Oxidative Metabolism of Phthalic Acid by Certain Soil Bacteria. By W. C. EVANS. (*Department of Agricultural Chemistry, Bangor*)

A gram-negative organism was isolated from soil and found capable of rapid and profuse growth, under aerobic conditions, in a phthalate (0·1%)–mineral salt medium.

Phthalate-grown cells were not adapted to 3-hydroxy- or 4-hydroxy-phthalate, *o*-, *m*-, or *p*-hydroxybenzoate, 2:3-, 2:4-, or 2:5-dihydroxybenzoate. It was also shown that these cells did not contain the enzyme system which converts catechol or *cis-cis*-muconic acid to *β*-ketoadipic acid. There was adaptation to protocatechuic acid.

Phthalate cultures, in the very early stages of growth only, gave a weakly positive colour reaction for an *o*-dihydric phenol. After concentration and solvent separation, it was shown by chromatography that the substance responsible for the above tests moved in an identical manner to 4:5-dihydroxyphthalic acid (nor-metahemipinic acid), and was different from 3:4-dihydroxyphthalic acid (nor-hemipinic acid). Sufficient of the intermediate has not yet been isolated for chemical characterization, neither has its subsequent fate in the culture been determined.

It would appear, however, that the initial step in the oxidative metabolism of phthalic acid by these soil organisms involves a di-hydroxylation process at the 4:5 positions of the aromatic ring.

[*The Editors of the Biochemical Journal accept no responsibility for the Reports of the Proceedings of the Society. Abstracts of papers read are published as received from the authors.*]

PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **343rd Meeting** *of the Biochemical Society was held in the Department of Biological Chemistry, Marischal College, Aberdeen, on Friday, 16th September, 1955, at 2.15 p.m., when the following papers were read:*

COMMUNICATIONS

Preliminary Experiments, using a Laboratory Rumen, on the Digestibility of the Carbohydrates in Grass at Different Stages of Growth.

By A. J. G. Barnett and R. L. Reid. (*Division of Agricultural Biochemistry, Department of Biological Chemistry, University of Aberdeen*)

Using a glass rumen of a pattern similar to that devised by Burroughs, Headley, Bethke & Gerlaugh (1950), attempts have been made to investigate pure cellulose and carbohydrate breakdown, as measured by volatile fatty acid formation, in the presence of filtered rumen liquor incorporating the nutrient salt medium of McDougall (1949) as modified by Burroughs *et al.* (1950). The total volatile fatty acid formation has been measured at intervals throughout periods of up to 70 hr., and the individual fatty acid pattern has been determined by the method of Duncan & Porteous (1953).

It has been shown that rumen fluid varies in its activity throughout the year, being of greatest potency in early summer. This physiological fact may account in part for the apparent diminution in the observed digestibility of late grass carbohydrate. The curves for total volatile fatty acid production in early grass are of a different type from those from late grass, a fact which may be related to the presence of easily utilizable soluble carbohydrate and fructosan in the former.

Again, concentrations of sulphatriazole in amounts corresponding to maximum and minimum oral dosage customarily given to the ruminant, result in a drop of over 90% in volatile fatty acid production, but from the experimental results obtained it would appear that the higher concentrations of the sulphonamide used were somewhat less effective in suppressing the acid formation than were the lower concentrations.

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The Estimation of 'Available Lysine' in Protein Concentrates.

By K. J. Carpenter and Gabrielle M. Ellinger. (*Rowett Research Institute, Aberdeen, Scotland*)

The reaction of fluoro-2:4-dinitrobenzene (FDNB) with the free ϵ-NH_2 groups of lysine in purified proteins, so that a stable, coloured DNP-lysine compound is formed on acid hydrolysis, has been used in studies of molecular structure (Sanger, 1945) and of the reaction of casein with carbohydrates (Lea & Hannan, 1950), where a reduction in the number of free ϵ-NH_2 groups was paralleled by a fall in nutritional value (Henry & Kon, 1950).

We have attempted to estimate the 'available lysine' in feeding-stuffs with a simplified method. Ground material containing 0·05 g. N is dispersed in 8 ml. 10% (w/v) $NaHCO_3$ in a 50 ml. round-bottom flask; 0·3 ml. FDNB, in 12 ml. EtOH, is added. After 2 hr. shaking EtOH is evaporated off, 24 ml. 5·5N-HCl are added and the whole refluxed for 24 hr. The cooled contents are filtered, with washings, and made to 50 ml. 5 ml. are extracted 4 times with 50 ml. ether, adjusted to pH 5 with 1 ml. glacial acetic acid and NaOH, diluted to 50 ml., centrifuged (if necessary) and read in the Spekker absorptiometer (filter 601). Replication without FDNB gives the 'blank'. ϵ-DNP-Lysine solutions provide a standard.

Results for fifteen samples of animal by-products (fish, whale and meat meals, casein, gelatin etc.) showed a highly significant correlation ($r = 0{\cdot}97$) with results obtained in a biological assay of protein quality with chicks (Carpenter, Ellinger & Shrimpton, 1955). In this assay materials are tested as supplements to cereal proteins which makes their lysine contribution particularly critical (Carpenter & Ellinger, unpublished).

These findings encourage further investigation of the FDNB reaction for the practical problem of

predicting 'protein quality' from the results of laboratory analyses. To claim that the method in its present form measures 'nutritionally available lysine' would involve the assumption that, with all the crude materials used, ϵ-DNP-lysine is the only compound formed that remains to give an extinction after acid hydrolysis and ether-extraction; also that the lysine molecules reacting with FDNB, and only those, are available to the chick. The high correlation with the biological assay results suggests that this may prove to be substantially true for the particular animal products tested. Preliminary studies indicate that the method cannot be applied to vegetable and fermentation products without modification.

Dr C. H. Lea and Dr S. M. Partridge kindly supplied ϵ-DNP-lysine.

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The Action of β-Amylase, Muscle Phosphorylase and Potato Phosphorylase on some Glycogens. By A. MARGARET LIDDLE and D. J. MANNERS. (*Department of Chemistry, University of Edinburgh*)

In continuation of structural studies on α-1:4-glucosans, a number of glycogens of known average chain length (determined by potassium periodate oxidation) have been degraded by β-amylase, and by muscle and potato phosphorylases. β-Amylase catalyses a stepwise hydrolysis of *alternate* linkages in exterior chains of branched α-1:4-glucosans, liberating maltose, whilst phosphorylases in the presence of inorganic phosphate, degrade *successive* linkages yielding glucose 1-phosphate. Since neither enzyme can hydrolyse or by-pass the 1:6 inter-chain linkages, the yield of maltose or glucose 1-phosphate is determined, to a first approximation, by the exterior chain length.

Twenty different glycogens had β-amylolysis limits in the range 25–56%, corresponding to β-limit dextrins (β-dextrins) with average chain lengths of 6–9. Ten different glycogens on treatment with muscle phosphorylase gave 14–35% conversion to glucose 1-phosphate, corresponding to muscle phosphorylase limit dextrins (ϕ-dextrins) with average chain lengths of 6–12. Glycogens which had been precipitated by 80% acetic acid and by ethanol had similar β-amylolysis and phosphorolysis limits (cf. Illingworth, Larner & Cori, 1952). Samples of waxy maize starch had β-amylolysis and phosphorolysis limits of 50–59% and 40–43% respectively.

A comparison of the average chain lengths of glycogen β- and ϕ-dextrins shows that the difference between them is not constant. We interpret this variation as an indication that different glycogens have different degrees of multiple-branching. Assuming (cf. Cori & Larner, 1951; Peat, Whelan & Thomas, 1952) that the A-chain 'stubs' of β- and ϕ-dextrins contain 2·5 and 1 glucose residue respectively, and that the corresponding B-chain 'stubs' contain n and $(n+4)$ residues, the above variation can be related to the ratio of A- and B-chains in the molecule, i.e. the degree of multiple-branching. (The most probable value of n is 2–3; for definition of A- and B-chains, see Peat, Whelan & Thomas (1952).) The calculated ratios ($\overline{AB}$) vary from approximately 1:1 (*Helix pomatia* glycogen) to 1:3 (foetal sheep liver glycogen).

Potato phosphorylase differs in action pattern from muscle phosphorylase since the former causes only approximately 10% degradation of 13-unit glycogens.

We are grateful to Professor E. L. Hirst, F.R.S., for his interest and encouragement.

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Observations on Barley β-Glucosidases. By D. J. MANNERS. (*Department of Chemistry, University of Edinburgh*)

Dillon & O'Colla (1950, 1951) have reported the occurrence of 'laminarinase' in wheat β-amylase preparations and in extracts of barley, oats, potato and hyacinth bulbs, whilst group specific β-glucosidase activity of an enzyme (*Z*-enzyme) isolated from soya-bean β-amylase preparations has been demonstrated by Peat, Thomas & Whelan (1952).

During an investigation of the action of amorphous barley β-amylase on glycogen (Bell & Manners, 1951), it was observed that β-1:3-glucosans (yeast glucan, laminarin) were also hydrolysed by this preparation. Crystalline sweet potato β-amylase, malt α-amylase and salivary α-amylase showed no 'glucanase' or 'laminarinase' activity. Accordingly, the barley β-amylase preparation was treated by the procedure devised by Peat *et al.* (1952) for the isolation of *Z*-enzyme from soya beans; the resulting preparation was free of amylolytic activity, but showed hydrolytic activity towards several β-glucosides.

β-Glucosidase activity has been followed by cuprimetric titration, paper chromatography and manometrically using glucose oxidase. The barley preparation hydrolysed aesculin, amygdalin, arbutin, cellobiose, cellodextrin, gentiobiose, helicin, laminarin, methyl β-glucoside, phenyl β-glucoside, phloridzin, salicin and yeast glucan. Cellodextrin and laminarin were hydrolysed in random fashion yielding glucose and a series of oligosaccharides. The 'laminarinase' activity was optimum at pH 5·0 (cf. Dillon & O'Colla, 1951) and was not inhibited by mercuric chloride (cf. Peat *et al.* 1952).

The available evidence indicates that barley contains at least three different enzymes catalysing the hydrolysis of β-glucosidic linkages, viz. a group specific β-glucosidase, an endo-β-1:4-glucosanase (cellulase) and an endo-β-1:3-glucosanase; the separation of these activities is being investigated.

During enzyme action on a concentrated solution of cellobiose, the formation of higher saccharides was observed; one of these appears to be cellotriose. The barley β-glucosidase preparation, unlike emulsin, cannot synthesize higher saccharides from glucose.

β-Amylase preparations from rye also show β-glucosidase activity (Liddle & Manners, unpublished).

I am grateful to Professor E. L. Hirst, F.R.S., for his advice and encouragement, and to Mr F. B. Anderson and Mr D. F. J. Kelly for experimental assistance.

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Changes in Carotenoid Synthesis in *Rhodospirillum rubrum* during Growth. By T. W. GOODWIN and M. E. SISSINS. (*Department of Biochemistry, The University of Liverpool*)

The major carotenoid pigment in mature *Rhodospirillum rubrum* is spirilloxanthin (van Niel & Smith, 1935; Polgar, van Niel & Zechmeister, 1944; Goodwin & Osman, 1953) and it has generally been assumed that the other pigments present in traces were *cis*-isomers of spirilloxanthin. Recently it was noted that the action spectrum for phototaxis in *Rsp. rubrum* (which is mediated by carotenoids) was different for young and old cultures; this led to the observation that the gross absorption spectrum of the carotenoid fraction of young (24 hr.) cultures was also different from that of older (3–6 day) cultures (van Niel, personal communication). These important findings prompted detailed investigation into the pigments present in young cultures and into the quantitative changes in pigment distribution during growth.

Young cultures (18–36 hr.) were found to contain lycopene, spirilloxanthin, rhodopin (?=lycoxanthin), 'demethylated spirilloxanthin', and two new pigments provisionally designated P 481 and hydroxy-P 481 (481 mμ. being in each case, the wavelength of the main absorption maximum in light petroleum, b.p. 40–60°). The relative amounts (percentage of total pigments present) of these pigments in 24 hr. cultures are, on average, 5·0, 28·8, 18·4, trace, 28·2 and 19·2 respectively. Maximal synthesis of all pigments except spirilloxanthin is observed in 24–36 hr. cultures. As the cells continue to multiply, the amounts of lycopene and lycoxanthin present decrease so that in 72–96 hr. cultures neither can be detected. P 481 and hydroxy-P 481 decrease more slowly during this period, whilst spirilloxanthin increases rapidly and continues to do so until the cultures are fully grown (120–144 hr. under our conditions), when it

represents about 95% of the total carotenoids, the remainder consisting of small amounts of P 481 and hydroxy-P 481.

It is interesting that young *Rsp. rubrum* cultures have the same qualitative distribution of carotenoids as have *Rhodopseudomonas palustris* (3 strains) and *Chromatium* spp. (Goodwin & Land, unpublished observations). *Rsp. rubrum* thus becomes unique amongst the Thio- and Athiorhodaceae (i.e. from the point of view of synthesizing almost exclusively spirilloxanthin) only when mature. None of the large number of photosynthetic bacteria so far examined shows any marked change in pigment synthesis during development, although minor changes are sometimes encountered.

We thank Dr C. B. van Niel for pointing out this problem to us and the Medical Research Council for financial assistance.

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The Relationship between Vitamin E and Cholesterol Metabolism. By H. J. Deuel, Jun., R. Cox, R. B. Alfin-Slater and B. H. Ershoff. (*Department of Biochemistry and Nutrition, University of Southern California School of Medicine, Los Angeles, California*)

The role of vitamin E in maintaining normal cholesterol levels in several species of animals has been suggested by various investigators (Morgulis & Spencer, 1936; Morgulis, Wilder, Spencer & Eppstein, 1938; Heinrich & Mattill, 1943) as a result of maintaining rabbits on a vitamin E-free diet with a subsequent production of elevated cholesterol levels in the blood and muscle. In this investigation, experiments were extended to include the guinea pig as well as the rabbit.

On synthetic diets, lacking vitamin E but adequate in all other respects, both guinea pig and rabbit developed the characteristic muscle paralysis accompanied by a sharp decrease in muscle creatine. Simultaneously there was an elevation in plasma cholesterol, muscle cholesterol and muscle lipids. The increase in plasma cholesterol in the guinea pig was mainly in the ester fraction; in rabbits both free and esterified cholesterol levels were elevated. The increase in total muscle lipids was much more marked in the guinea pig than in the rabbit. The increased cholesterol levels in the muscle of both species were principally as free cholesterol. No changes were noted in either total lipid or cholesterol content of the liver. In the rat, however, the absence of vitamin E from diets containing 1% cholesterol gave rise to an increased amount of esterified cholesterol in the liver as compared with the liver of rats fed a cholesterol diet adequate in this vitamin.

This investigation has been supported by a grant from the Muscular Dystrophy Associations of America.

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The Permeability of the Goat Placenta to Sugars. By D. G. Walker and F. L. Warren. (*Department of Biochemistry, London Hospital Medical College, London, E.* 1)

Experiments similar to those of Huggett, Warren & Warren (1951) on sheep have been performed on pregnant goats. The results indicate that in this species also, where fructose is the chief sugar of the foetal blood and fluids, the site of fructose formation is the placenta, the precursor being the foetal blood glucose.

The fact that fructose occurs in the foetal blood but not in the maternal blood suggests that the placenta is impermeable to fructose. When fructose was injected intravenously into the maternal circulation there was no rapid transfer to the foetus; the foetal blood fructose increased at only a slow rate similar to that obtained following glucose infusion into the maternal circulation. Glucose, however, passed rapidly across the placental barrier in both directions.

Other sugars were also injected into the maternal (via jugular vein) and foetal (via umbilical cord) circulations and their fates studied by following the total reducing sugars, fructose, and pentose, in Ba/Zn protein-free blood filtrates. This information was supplemented by paper chromatography of free sugars in selected blood filtrates.

Mannose passed rapidly across the placenta in both directions. A small but significant rise in the foetal blood fructose occurred when mannose was injected into the maternal circulation. The placenta was also freely permeable to galactose and to xylose. A small amount of sorbose appeared to traverse the placenta. This amount was just large enough to be detected chromatographically. The goat placenta was impermeable to reducing (maltose and lactose) and non-reducing (sucrose) disaccharides.

These differences in permeability are at present difficult to explain. The rapid transfer of glucose, galactose, mannose, and xylose suggests that a rapid passive diffusion of these sugars can occur. The slow passage of fructose from mother to foetus suggests the possibility of an active process of transfer (presumably enzymic) and this may be similar to the mechanism of conversion of glucose into fructose by the placenta.

We are grateful to Dr H. Harris for the chromatographic work and to the Medical Research Council for a grant towards expenses.

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Accumulation of Pyruvate and Citrate During Growth of a vibrio with Fluorine Compounds present. By S. Dagley and J. R. L. Walker. (*Department of Biochemistry, University of Leeds*)

When a non-proliferating suspension of a vibrio was aerated with benzoic acid, α-oxoglutarate accumulated; the compound disappeared from the medium when growth resumed on addition of ammonium sulphate as source of nitrogen. When sodium fluoroacetate (5 mM) was present during growth, no α-oxoglutarate was detected; instead, pyruvate was produced in amounts sufficient for isolation as the 2:4-dinitrophenylhydrazone. Citrate also accumulated and was estimated by the method of Taylor (1953). Production of both citrate and pyruvate during growth at the expense of several other sources of carbon was demonstrated. This observation is of particular interest in the case of acetate, where C-2 units gave rise to C-6 units, since it focuses attention once more upon the unsolved problem of how oxaloacetate is produced from C-2 units in such systems. The level of pyruvate attained when growth ceased was dependent on the carbon source. When succinic, malic, fumaric, benzoic or *p*-hydroxybenzoic acids were utilized 0·5–1·0 μmole/ml. accumulated, but acetic, octanoic and sebacic acids gave rise only to about one-tenth of this amount.

Gal, Peters & Wakelin (1954) have reported *in vitro* inhibition of pyruvate oxidation (brain) by fluoropyruvate. When this compound was present in growing cultures at 10^{-4}M, pyruvate accumulated but citrate did not; and with acetate as substrate much smaller amounts were given than with benzoate. The following tentative suggestions may be made to account for our observations. For micro-organisms as distinct from other cells, terminal respiratory processes may include a direct route from acetate to pyruvate and 'lethal synthesis' of fluoropyruvate as well as fluorocitrate may occur when fluoroacetate is added to growing cultures. Partial blockage of pyruvate oxidation by fluoropyruvate may cause accumulation of pyruvate if the latter is produced chiefly from C-4 units, as when dicarboxylic and aromatic acids are substrates. On the other hand only accumulation of citrate may be observed if growing cultures produce pyruvate from acetate by a reaction significantly slower than those which produce citrate.

Our demonstration of citrate synthesis during utilization of a variety of aromatic and long-chain fatty acids suggests that widely divergent metabolic routes converge upon the Krebs cycle.

We thank Dr J. Mager for a gift of fluoropyruvic acid.

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The Origin of the Trisaccharide Melezitose. By J. S. D. Bacon and Brenda Dickinson. (*Department of Biochemistry, University of Sheffield*)

The honeydews and mannas which contain melezitose (3-α-D-glucopyranosyl-β-D-fructofuranosyl α-D-glucopyranoside; see Hehre, 1953) have been discussed by Hudson (1946): all may reasonably be suspected of being derived from plant tissue by the action of 'sucking' insects (Hemiptera). Hudson, like earlier authors (cf. Maquenne, 1893), thought it improbable that the trisaccharide was made by insects, and accordingly surmised that melezitose is a constituent of the tissues of plants of widely different genera.

The discovery that honey invertase has transglucosylase activity (White, 1952) offered an alternative explanation: that incomplete digestion

of sucrose in the gut of an aphis might lead to the excretion of trisaccharides, including melezitose (cf. Duspiva, 1953). However, White & Maher (1953*a*, *b*) reported that 'maltosyl-fructoside' was the main product of honey invertase, and Gray & Fraenkel (1953) later found it in the honeydew of *Pseudococcus citri* Risso feeding on potato sprouts: melezitose was not reported to be present in either case.

From a study of lime trees, *Tilia* spp., and aphids, *Eucallipterus tiliae* L., feeding upon their foliage, we have come to the conclusion that melezitose may nevertheless be an insect product. Extracts of the leaves of the trees, which were rich in sucrose, contained no melezitose detectable on paper chromatograms when about 1·5 mg. of extractable solids were applied to each spot. In contrast, as Maquenne (1893) observed, the honeydew from the leaves contained appreciable amounts of melezitose; 1·0 g. dry weight of honeydew yielded 300 mg. of crude, 244 mg. of crystalline, trisaccharide. Furthermore, the crushed insects, when incubated with sucrose solution, liberated glucose and fructose and formed trisaccharide; 67 mg. of insects in 2·5 ml. 20% (w/v) sucrose incubated for 3 days at 22°, in the presence of chloroform, yielded about 35 mg. of a trisaccharide fraction which crystallized from aqueous ethanol when seeded with melezitose. The R_F values and colour reactions of this substance and its mild acid hydrolysis products were identical with those of melezitose. Crystals of the two substances were similar in appearance, and X-ray powder photographs (by Mr O. G. Griffin of the Safety in Mines Research Establishment, Sheffield) were almost identical.

In view of the large amounts formed in relation to the fresh weight of insects taken it seems likely that sucrose is the sole substrate for melezitose synthesis.

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Non-protein Nitrogenous Constituents of Locust Muscle. By W. O. Kermack and Janet M. Stein. (*Department of Biological Chemistry, University of Aberdeen*)

The non-protein nitrogenous constituents of the wing muscles of the African migratory locust (*Locusta migratoria migratorioides*) have been investigated. The muscle was washed with 1% saline before removal, to avoid gross contamination with haemolymph, and then twice extracted with 5% trichloroacetic acid. In a typical muscle extract from male locusts, 15 days after the final moult, the nitrogenous constituents, in mg. N/100 g. muscle were as follows: total N, 398; α-amino N (Pope & Stevens, 1939), 187; volatile amine N, 19; glutamine N, 9; adenylic acid N, 56; uric acid N, 0. Of the total non-protein N, 127 mg./100 g. muscle thus remain unaccounted for. Some of this is due to taurine, and some to the guanidyl group of arginine. Quantitative amino-acid estimations indicate that arginine is an abundant amino-acid, and this is probably mainly derived from the breakdown of the arginine phosphagen. The non-protein nitrogenous constituents have been found to vary with the age, sex and physiological state of the locust.

Amino acids were examined by the technique of two-dimensional paper chromatography. Sixteen amino acids were detected in trichloroacetic acid extracts, fourteen being identified, of which arginine, glutamine and glycine were most abundant. Taurine also was present in considerable quantities. Eighteen amino acids have been identified in muscle hydrolysates and the evidence suggests that one of these is lanthionine (Stein, 1955).

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The Selective Inhibition of Glycosidases by the Corresponding Aldonolactones. By J. Conchie and G. A. Levvy. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

Following the discovery that β-glucuronidase is inhibited by saccharo-1:4-lactone (Levvy, 1952), and β-glucosidase by glucono-1:4-lactone and glucono-1:5-lactone (Conchie, 1954), it was decided to investigate the possibility that as a general rule β-glycosidases, and perhaps also α-glycosidases, are inhibited by the aldonolactones corresponding to the substrates in the configuration of the secondary hydroxyl groups. A large number of sugar acid lactones are in the course of being tested as in-

hibitors for a variety of glycosidases, mainly obtained from the rumen of the sheep. The enzymes already examined include α- and β-glucosidase, α- and β-galactosidase, and β-xylosidase. Powerful and selective inhibition of the β-glycosidases was observed with the aldonolactones derived from the same parent sugars as the substrates. α-Glucosidase was as powerfully inhibited by the gluconolactones as β-glucosidase, but α-galactosidase was only feebly affected by galactono-1:4-lactone, and by no other compound tested.

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A Method for the Estimation of Starch and Sugars in Cereals using the Anthrone Reagent.

By K. M. Clegg. (*Rowett Research Institute, Bucksburn, Aberdeenshire*)

The colour reaction of anthrone with carbohydrates has been adapted to give a simple and rapid method for the estimation of the starch and sugar content of cereals and cereal by-products. The sugars are extracted from 0·2 g. of the cereal with 80% ethanol and the starch in the residual material is brought into solution with 52% perchloric acid (McCready, Guggolz, Silveira & Owens, 1950). The ethanol is removed from the extract of sugars, and then the starch and sugar extracts are treated in a similar manner. They are diluted to contain approximately 100 μg. sugar/ml. and 1 ml. is used for the analysis. The colour complex, which has maximum absorption at 620 mμ., is developed with the anthrone reagent according to the modification of Fairbairn (1953). An internal glucose standard of 100 μg. is used, and the conversion factor of 0·9 is applied for the estimation of the starch extract.

Results for the starch content of cereals and cereal by-products showed a standard deviation of 2·2% of the mean. The results have been of practical value in assessing the metabolizable energy value of feeding stuffs for poultry (K. J. Carpenter & K. M. Clegg, unpublished results). The method has also been applied to the starch and sugar analysis of mixed poultry rations; the presence of non-cereal material gave no interference.

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PROCEEDINGS OF THE BIOCHEMICAL SOCIETY

The **344th Meeting** *of the Biochemical Society was held in the Department of Physiology, Bedford College, Inner Circle, Regent's Park, N.W.* 1, *on* 22 *October* 1955, *at* 2.00 *p.m. when the following papers were read:*

COMMUNICATIONS

The Effect of Adenosine on the Movement of Sodium between Erythrocytes and the Suspension Medium. By E. J. Harris and T. A. J. Prankerd. (*Department of Biophysics, University College, and Medical Unit, University College Hospital Medical School, London*)

The rates of entry and extrusion of the major part of the Na of red cells depend upon the condition of the cells. Storage *in vitro* overnight leads to diminution of both rates (E. J. Harris & Prankerd, 1953). Since Prankerd & Altman (1954) have shown that the addition of adenosine to a suspension will maintain or even lead to restoration of the cell organic phosphates it seemed likely that Na movement might also be affected by this substance.

When added to a suspension of fresh cells in a diluted plasma containing labelled Na, 0·005 M adenosine in some experiments had no effect, and in some caused an increase in cell Na, without changing the rate of output of the main part of the cell Na (the cell Na does not behave homogeneously). For example, with glucose the inward rate constant was 0·065 $hr.^{-1}$, and outward rate constant 0·35 $hr.^{-1}$, with adenosine the figures were 0·092 and 0·36 $hr.^{-1}$ respectively. The effect on cells which had been kept *in vitro* was upon both inward and outward rate constants. For example, using the same batch of cells: with glucose—inward 0·031, outward 0·176 $hr.^{-1}$; with adenosine—inward 0·126, outward 0·374 $hr.^{-1}$. In another experiment, using a different batch of cells, the figures were: with glucose—inward 0·025, outward 0·072 $hr.^{-1}$; with adenosine—inward 0·034, outward 0·125 $hr.^{-1}$.

The adenosine added to the suspension is degraded to adenine, and this occurs whether or not additional glucose is added. Cells incubated with ouabain (10 μg./ml.) gain Na and lose K (as found for cardiac glycosides and aglycones by Schatzmann, 1953), but the presence of adenosine protects the cells from this effect. Thus there is evidence that ion movements can be potentiated either by glucose (J. E. Harris, 1941) or by adenosine, and that the latter substance better maintains the outward Na movement of stored cells.

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The Interaction of Proteins and Aminostilbenes. By D. F. Cheesman. (*Department of Physiology, Bedford College, University of London*)

It has been reported (Cheesman, 1954) that those compounds related to 4-aminostilbene which have a nucleotoxic action (Haddow, Harris, Kon & Roe, 1948) produce an apparent cross-linking effect on unimolecular films of the protein globin, in that they lower the gelation pressure of the films and greatly increase the mechanical strength of protein fibres drawn from the surface.

Experiments have been made to elucidate the mechanism of this action, which was scarcely to be expected with compounds containing a single polar group. Films were spread on the Langmuir trough from solutions in 80% (v/v) *iso*propanol of beef globin hydrochloride (0·4 mg./ml.) with and without admixed 4-dimethylamino-2′-methyl-*trans*-stilbene (DAMS, 0·1 mg./ml.). The substrate was 0·02 M-Na_2HPO_4-KH_2PO_4 buffer. The gelation pressure was determined by observation of the movements of talc particles floating in the surface.

It has been established that the reduction in gelation pressure (Δp) caused by DAMS is highly dependent upon pH, showing a pronounced maximum at pH 6·7, about which the Δp/pH curve is symmetrical. This maximum corresponds approximately to the isoelectric point of native globin. When the globin solution is boiled or aged before spreading, the maximum is flattened and displaced to pH 6·9. A similar displacement of the isoelectric point is to be expected (cf. Putnam, 1953). The film gelation points of zein and gliadin, proteins without free carboxyl groups, are unaffected by DAMS.

These observations suggest that an equivalence of ionized basic and carboxyl groups in the protein

favours the cross-linking action, and hence that the active aminostilbene molecules are strongly polarized by interaction with suitably disposed ion pairs on the protein surface. The cross-linking would then involve the establishment of electrovalent bonds between the aminostilbene N atom and a protein carboxyl group, and between, say, the 4′-C atom of the aminostilbene and a protein basic group.

Such a polarization, which would involve an increased contribution of quinonoid structures to the molecule, might be expected to cause a bathochromic shift in the ultraviolet absorption spectrum of the aminostilbene. Differential spectrometry on mixed solutions of native beef globin (0·2 mg./ml.) and DAMS (0·5 μg./ml.) showed this shift to be very marked (5–15 mμ.) in the pH range 4–9. It is accompanied in the central part of this range by a maximum or an inflexion at about 345 mμ., which is absent or less pronounced in the absence of protein, although the principal maximum appears near this wavelength for solutions of DAMS in ethanol (Haddow *et al.* 1948; Beale & Roe, 1952).

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The Enzymic Interconversion of Pyridoxal Phosphate and Pyridoxamine Phosphate by Aqueous Extracts of *Escherichia coli*. By R. B. Beechey and F. C. Happold. (*Department of Biochemistry, University of Leeds*)

In unpublished work the authors have shown that, over a wide range of conditions, pyridoxal phosphate is the only member of the vitamin B_6 group that will activate apotryptophanase. However, on incubation of pyridoxamine phosphate with aqueous extracts of apotryptophanase at pH 8·2, activation of the apotryptophanase occurs. In the presence of Mg^{2+}, optimal concentration 2×10^{-3} M, this effect was greatly enhanced. Other divalent metal ions, i.e. Mn^{2+}, Co^{2+}, Zn^{2+}, Fe^{2+} and Cu^{2+}, showed no activating effect. These results suggested the conversion of pyridoxamine phosphate to pyridoxal phosphate.

Peaks in the ultraviolet absorption spectrum of pyridoxal phosphate and pyridoxamine phosphate, at pH 8·2, occur at 390 and 320 mμ. respectively. The enzymic conversion of pyridoxamine phosphate to pyridoxal phosphate was confirmed by following the decrease in optical density at 320 mμ. and the simultaneous increase at 390 mμ. which occurred when aqueous *Escherichia coli* extracts were incubated with pyridoxamine phosphate at 37°, pH 8·2. Similarly, the conversion of pyridoxal phosphate to pyridoxamine phosphate was demonstrated by following the decrease in optical density at 390 mμ. and the simultaneous increase at 320 mμ. Mg^{2+} appears to accelerate only the conversion of pyridoxamine phosphate to pyridoxal phosphate.

Incorporation of [^{14}C] Amino Acids into Rat Liver and Hepatoma Proteins *in vitro*. By P. N. Campbell and Nancy E. Stone. (*Courtauld Institute of Biochemistry, Middlesex Hospital, London, W.* 1)

An attempt has been made to follow the synthesis of a specific protein, serum albumin, in slices of rat liver and hepatoma, and to compare the rate of its synthesis with that of the other proteins in the two tissues.

The method of Peters & Anfinsen (1950) for following the synthesis of albumin in chick liver slices has been adapted. Rat serum albumin was purified by ammonium sulphate precipitation, followed by zone electrophoresis on cellulose columns (Porath, 1954). Antiserum was obtained by injecting an alum precipitate of the albumin into rabbits. Hepatomas were produced in rats by including *p*-dimethylaminoazobenzene in the diet (Kline, Miller, Rusch & Baumann, 1946).

Slices, obtained from the non-cancerous lobes of livers bearing small hepatomas and from the hepatomas, were incubated aerobically in a medium containing [^{14}C]glycine for periods up to 4 hr. After incubation the slices were removed from the medium and homogenized in buffer. A clear supernatant was obtained from the homogenate by centrifugation. Albumin was precipitated from the incubation medium and from the supernatant by addition of antiserum. The total protein in medium and supernatant was precipitated with trichloroacetic acid. Comparison of the radioactivity of these protein fractions showed that whereas the radioactivity of the trichloroacetic acid precipitate from hepatoma

was much greater than that from liver, the hepatoma albumin was slightly less active than the liver albumin.

These results suggest that the higher rate of incorporation of radioactive amino acids into the soluble proteins of hepatoma compared with liver cannot be explained by differences in the sizes of the free amino acid pools in the two tissues. Thus if incorporation of radioactive amino acids can be interpreted as a measure of protein synthesis, it may be concluded that in general the synthesis of proteins in hepatoma slices is more rapid than that in liver slices.

When the slices were incubated with [^{14}C]glucose instead of [^{14}C]glycine both the albumin and the trichloroacetic acid precipitated protein of the hepatoma were more radioactive than those from liver. This difference can be accounted for on the basis that the dilution of [^{14}C]glucose by inactive intracellular glucose is greater in the case of liver slices than it is in hepatoma slices.

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Effect of 2:4:6-Triethyleneimino-1:3:5-triazine on Carbohydrate Metabolism in Ascites Tumour Cells. By I. M. Roitt. (*Courtauld Institute of Biochemistry, Middlesex Hospital, London, W.* 1)

The potent growth-inhibitory properties of the ethyleneimines suggested that a study of the interaction of these compounds—in particular 2:4:6-triethyleneimino-1:3:5-triazine (TEM)—with carbohydrate metabolism in normal and malignant mammalian cells would be of interest.

Anaerobic glycolysis in Krebs mouse ascites tumour cells was completely inhibited by 0·001 M TEM. Higher concentrations were required to inhibit anaerobic glycolysis in slices of rat brain cortex, spleen and kidney cortex, but the sensitivity of liver and hepatoma to the inhibitor was of the same order as that of the ascites tumour. Respiration in ascites tumour cells, brain and kidney slices was also affected by TEM. A marked delay in the onset of these inhibitions was prominent, and was not due to slow penetration of the inhibitor into the cells or to the occurrence of a slow reaction of TEM with the Krebs Ringer medium to yield an inhibitory substance.

Since TEM reacts with the sulphydryl groups of cysteine, glutathione and β-mercaptoethylamine, its effect on the activity of the 'SH-enzymes' hexokinase, triosephosphate dehydrogenase and phosphofructokinase was studied. Only the latter was strongly inhibited. However, analysis of phosphorylated intermediates (LePage, 1949) in ascites tumour cells which had been inhibited by TEM under anaerobic conditions revealed a greater accumulation of fructose diphosphate than of the monophosphate; triosephosphate concentration was higher in the inhibited cells than in the controls but phosphoglyceric acid concentration was unaltered. Although it was shown that extracts of the TEM-inhibited cells contained active triosephosphate and lactic acid dehydrogenases, addition of pyruvate to the inhibited cells failed to restore glycolysis, suggesting that the concentration of DPN was inadequate.

Concurrent with the onset of inhibition, the DPN concentration fell to less than 20% of the control value and no DPN could be detected in the surrounding medium. This rate of fall was much higher than that observed when glycolysis was arrested by anaerobic incubation of the cells at 38° in the absence of glucose. There was no direct reaction between DPN and TEM. Nicotinamide afforded protection against the inhibitory action of TEM and could also reverse the inhibition. The low concentration of DPN appears to be the cause rather than the consequence of the inhibition by TEM, the glycolytic enzymes themselves remaining seemingly unaffected.

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The Effects of Glutathione on Potassium Toxicity in Rats. By D. F. Cole. (*Department of Medicine, University of Durham, Medical School, Newcastle-on-Tyne* 1)

Subcutaneously injected glutathione increases the resistance of both intact and adrenalectomized mice to potassium poisoning (Zwemer, Vollmer & Carey, 1951; Martorano, Zwemer & Vollmer, 1953). It seemed possible that the protection against potassium poisoning afforded by glutathione and that which is caused by adrenal cortical steroids (Zwemer & Truszkowski, 1936; Truszkowski & Duszynska, 1940) might show common features. We have therefore studied renal excretion

of potassium, and potassium concentrations in skeletal and cardiac muscle after a single injection of potassium chloride, given to rats either with or without previous glutathione treatment. The effects of previous adrenalectomy and of feeding high- or low-potassium diets were also investigated.

We confirmed the protective action of glutathione, but did not observe any increase of renal potassium excretion nor did its action depend on preventing any increase of skeletal or cardiac muscle potassium. In these respects glutathione protection differs fundamentally from the action of deoxycorticosterone or adrenal cortical extract (Ferrebee, Parker, Carnes, Gerity, Atchley & Loeb, 1941; Harkness, Muntwyler, Mautz & Mellors, 1942). Although intact rats given high-potassium diets showed values for tissue potassium as high, or higher than, adrenalectomized rats, signs of potassium poisoning were evident only in the adrenalectomized series. This suggests that the appearance of toxic effects is not entirely dependent on tissue concentrations of potassium. It is also suggested that glutathione protection depends on alterations of tissue metabolism dissimilar to those caused by mineralo-corticoids. Recently, Booker, Tureman, Dacosta, Poulson & Mitchell (1955) also concluded that glutathione and cortisone differ in the manner in which they protect mice against cold-stress.

This work was carried out during the tenure of an I.C.I. Research Fellowship.

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A Comparison of the Dry Weights of Isolated Cell Nuclei, as determined by Chemical and by Interferometric Methods. By A. J. Hale and E. R. M. Kay (introduced by J. N. Davidson). (*Institute of Physiology and Department of Biochemistry, The University, Glasgow*)

The amount of certain materials present in isolated cell nuclei depends upon the procedure used to isolate these nuclei (Dounce, 1955). It is not known whether the mean dry weight of millions of nuclei, as determined by bulk chemical methods, is the same as the mean dry weight of tens, as determined by interferometric microscopy, or whether the method of isolation affects the result.

The nuclei of calf's thymus were isolated (*a*) by the citric acid method (CN) of Mirsky & Pollister (1946), (*b*) by the non-aqueous method (NAN) of Kay, Smellie, Humphrey & Davidson (1955), and (*c*) by the sucrose-calcium chloride method (SN) of Schneider & Peterman (1950).

The chemical determination made use of the method described by Smellie, Humphrey, Kay & Davidson (1955) for the estimation of deoxyribonucleic acid phosphorus (DNA-P) and ribonucleic acid phosphorus (RNA-P). The mean dry weight of the CN and SN were estimated by counting the nuclei in an aliquot of each using a haemocytometer, weighing the dry residue of a similar aliquot and thereby calculating the dry weight per nucleus. The mean dry weight of NAN was estimated by measuring the DNA-P content of a known weight of NAN and calculating the number of nuclei present by dividing the total DNA-P by the mean DNA-P per nucleus obtained from the CN, and then dividing the known weight of the sample by the number of nuclei present.

Interferometric determination of the dry weight of individual nuclei was made by the trace displacement method described by Davies, Wilkins, Chayen & LaCour (1954). Approximately thirty-five nuclei were examined in each sample by this method.

The mean dry weight of the CN was 19·1 and 19·8 pg. as determined by chemical and interferometric methods respectively. The corresponding values for SN were 23·0 and 21·1 pg. and those for NAN were 33·0 and 35·2 pg. The interferometric method showed that there was a considerable scatter about the mean values in all samples.

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Uptake of B_{12}-Vitamins in *Ochromonas malhamensis*. By J. E. Ford, Margaret E. Gregory and E. S. Holdsworth. (*The National Institute for Research in Dairying, University of Reading*)

The protozoan *Ochromonas malhamensis*, like certain higher animals, is narrowly selective in its requirement for 'vitamin B_{12}'. Of the naturally occurring B_{12} vitamins, only cobalamin and vitamin B_{12III} are active (cf. Coates & Ford, 1955), and under conditions specified for the assay of vitamin B_{12} with this micro-organism the other vitamin B_{12}-like compounds do not interfere.

In a culture of *Ochromonas* grown with limiting cyanocobalamin, added cyanocobalamin, vitamin B_{12III}, pseudovitamin B_{12} or factor A were taken up by the cells in about the same amount. Factor B was taken up very little, if at all.

Above a certain critical concentration in the culture liquor, the rate of uptake of added cyanocobalamin increased sharply. Furthermore, the uptake of cyanocobalamin added at a concentration (0·0005 μg./ml. of culture) below this critical level was progressively increased by the simultaneous addition of pseudovitamin B_{12} or factor A in concentrations between 0·001 and 0·006 μg./ml. At higher concentrations the uptake of cyanocobalamin was increasingly inhibited. Factor B was without effect.

On measuring the rate of the growth response to a limiting concentration of cyanocobalamin, we found that at low levels ($<0{\cdot}02$ μg./ml. culture medium) pseudovitamin B_{12} and factor A were slightly stimulatory, but at higher concentrations they retarded growth. Again, factor B was without effect.

In more detailed experiments with pseudovitamin B_{12} we established that, except at relatively low concentration, the analogue inhibits competitively the growth response to cyanocobalamin.

Within a wide range of concentration the influence of pseudovitamin B_{12} and factor A was manifest only in the *rate* of growth. The peak cell population densities attained in the cultures were determined by the limiting concentrations of cyanocobalamin supplied in the medium.

These findings may be linked with our observation that extracts of *Ochromonas* cells contain a substance which combines with cyanocobalamin, factor A or pseudovitamin B_{12} and renders them non-ultrafiltrable through cellophane. The same or a similar substance appears also in the culture liquors, more especially in those from aged cultures.

Culture liquors of *Euglena gracilis* also have the property of sequestering vitamin B_{12}. This may be connected with Kristensen's (1955) finding that *Euglena* culture liquors contain a thermolabile factor which inhibits growth of the organism in a vitamin B_{12} limiting medium.

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A Block in Arginine Synthesis in *Neurospora crassa* due to Gene Mutation. By J. R. S. Fincham and J. B. Boylen. (*Department of Genetics, University College of Leicester*)

Arginine-requiring mutant strains of *Neurospora crassa* were first studied by Srb & Horowitz (1944). These authors described one type of mutant which required arginine specifically, failing to respond to citrulline. A second such mutant type, genetically different from the first, has recently been discovered (D. Newmeyer, personal communication), and the present paper is concerned with the biochemical characteristics of this type.

Four strains, which so far appear to be identical, have been studied. All of them were found to accumulate argininosuccinic acid in the mycelium, and this accumulation was considerably enhanced by the addition of citrulline to the growth medium. Citrulline added to the mutant cultures could be almost quantitatively accounted for at the end of growth as residual citrulline plus argininosuccinic acid, while citrulline added to parallel wild-type

cultures completely disappeared. This accumulation of argininosuccinic acid by mutant mycelium in the presence of citrulline was accompanied by a marked depletion of the amino acids (predominantly alanine, glutamine and glutamic acid) normally present in mycelium.

Cell-free extracts of wild-type mycelium were found to contain an enzyme catalysing the reversible splitting of argininosuccinic acid to arginine and fumaric acid. This enzyme was purified fivefold by ammonium sulphate fractionation. The mutant strains appeared to lack this enzyme; no activity could be found either in crude extracts or in ammonium sulphate fractions, although as little as 0·2% of the normal wild-type activity should have been detectable if present. Extracts of the mutant strains contained no detectable inhibitor of the enzyme. Arginase and fumarase were produced by the mutants in normal amounts. This case appears to be a straightforward one of a gene mutation having its effect through affecting the production of a single enzyme.

The results indicate that the mechanism of the conversion of citrulline to arginine in *N. crassa* is similar to that demonstrated by Ratner and her collaborators (Ratner, Petrack & Rochovansky, 1953; Ratner, Anslow & Petrack, 1953) for mammalian tissues.

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The Detection of 16-Oxooestradiol-17β in the Urine of Pregnant Women. By Elizabeth J. D. Watson and G. F. Marrian. (*Department of Biochemistry, University of Edinburgh*)

Following the isolation of 16-*epi*oestriol from the urine of pregnant women, Marrian & Bauld (1955) tentatively suggested that the common metabolic precursor of both *epi*oestriol and oestriol might be 16-oxooestradiol-17β (cf. Huffman & Grollman, 1947). A systematic search for 16-oxooestradiol-17β in the urine of pregnant women has now been conducted.

Ether-soluble 'neutral and phenolic' fractions from acid-hydrolysed pregnancy urine were treated with Girard's Reagent T by refluxing in ethanol-acetic acid solution. From the ketonic fractions thus obtained the phenolic-ketonic fractions were separated in the usual way. Such phenolic-ketonic fractions were partitioned on Celite columns with 70% methanol as stationary phase and benzene-ethylene dichloride (75:25) as mobile phase.

These chromatograms revealed the presence in the urinary extracts of two different ketonic Kober-chromogens. The elution pattern of the less 'polar' of these was indistinguishable from that of oestrone in the same solvent system, while the elution pattern of the more 'polar' one was indistinguishable from that of synthetic 16-oxooestradiol-17β prepared by the method of Huffman & Lott (1948). In three experiments on different samples of late pregnancy urine the yields obtained of the 'more polar' Kober-chromogen were 81, 307 and 307 μg./24 hr. respectively.

The phenolic-ketonic fraction from 200 l. of late pregnancy urine was subjected to a 10-transfer countercurrent distribution in separating funnels using the solvent system 50% ethanol-benzene: hexane (80:20). The observed distribution of Kober-chromogens closely resembled the theoretical distribution for a mixture of oestrone and 16-oxooestradiol-17β which was calculated from the determined partition ratios of these compounds (oestrone, K = 9·8; 16-oxooestradiol-17β, K = 0·95).

In view of this evidence it is believed that the 'more polar' ketonic Kober-chromogen is identical with 16-oxooestradiol-17β.

Yields of 16-oxooestradiol-17β from late pregnancy urine were increased 2- to 3-fold by substituting incubation at pH 4·6 with an enzyme preparation from *Patella vulgata* for the usual preliminary acid hydrolysis of the urine. Subsequently it was found that although 16-oxooestradiol-17β is stable to boiling for 60 min. with 15 vol. % of 10N-HCl in aqueous solution, it undergoes extensive destruction when subjected to the same treatment after solution in urine. It was also found that 16-oxooestradiol-17β undergoes extensive destruction when heated in ethanolic solution with acetic acid and Girard's Reagent T. It is concluded, therefore, that the amounts of 16-oxooestradiol-17β determined in the early experiments must have represented only small fractions of those that were actually present in the urine.

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The Reaction of L-Histidine with D-Glucose. By S. LEWIN. (*Physical Chemistry Laboratory, South-West Essex Technical College, London, E.* 17)

The reaction of histidine with glucose has been noted (Ågren, 1940; Kubota, 1941), but no systematic investigation was carried out to determine its mechanism.

We have investigated, aerobically and anaerobically, this combination using varying concentrations (M/15 to M/62·5 L-histidine, and up to 1·2M D-glucose) over the pH range 5–9, and at various temperatures (20°, 30°, 40° and 50°) by following the resulting pH depressions using glass electrode measurements.

As soon as the glucose and histidine solutions were mixed, the pH of the mixture dropped, eventually reaching a steady value. This was reached within a week at 20° and at the higher temperatures in shorter periods, requiring less than 2 hr. at 50°. The results obtained in this steady pH state, over the approximate pH range of 8–9, have been found to be in accord with a mechanism involving one glucose molecule and one anionic histidine molecule, similar to that proposed by Katchalsky (1941) for several other amino acid-glucose combinations. No combination was detected over the pH range of 5–6·5. The value of the equilibrium constant decreased with rise in temperature from 3·9 at 20° to 2·3 (±0·2) at 50° respectively, thus showing the reaction to be exothermic.

The life-time of the above first steady pH state decreased with rise in temperature; it lasted about 4 weeks at 20°, but only about 5 hr. at 50°. It was followed by a further pH drop leading eventually to a second steady pH state. At the higher temperatures several consecutive steady pH states were found.

The development of brown colour, as associated with the 'browning reaction', was observed visually at the end of the second, or the beginning of the third, steady pH state, and its intensity increased with the passage of time. Less intensity of colour was found under aerobic than under anaerobic conditions. Increase in initial pH and glucose concentration resulted in the browning becoming more pronounced. No browning was noted even after 8 months when the glucose-histidine mixtures were kept below 0°.

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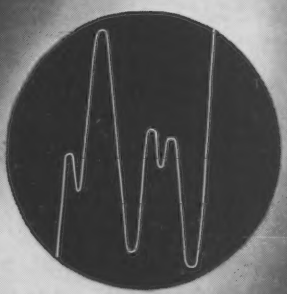

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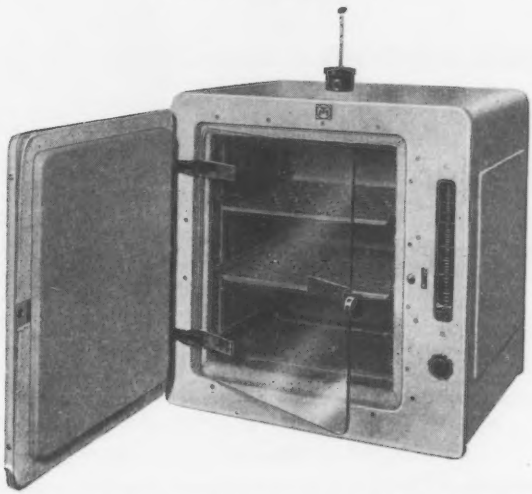

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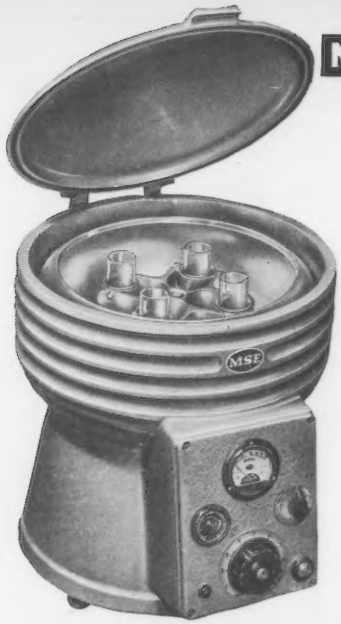

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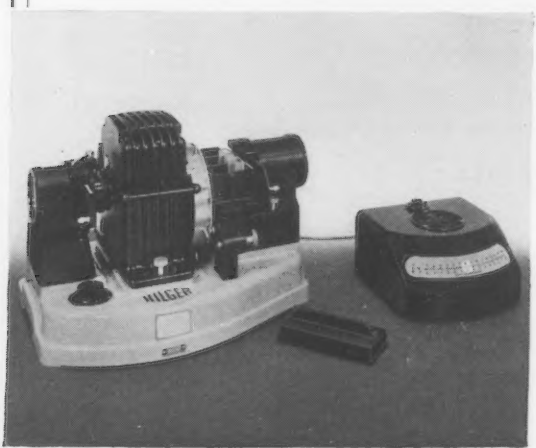

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